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ORIGINAL ARTICLES

The Immunomodulating Effect of Theophylline on Lymphocytes from Chronic Lymphocytic Leukemia Patients

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SUMMARY **The *in vitro* immunomodulating effects of theophylline on E-rosette formation, phytohemagglutinin (PHA) response, and Ig surface receptors of B lymphocytes were studied on fresh as well as on preincubated lymphocytes from patients with B cell chronic lymphocytic leukemia (CLL). In 11 out of 14 CLL patients, 24 hours preincubation at 37°C significantly enhanced E-rosette formation. Subsequent treatment of preincubated cells with appropriate concentrations of theophylline further enhanced E-rosette formation in 11 cases. On fresh lymphocytes the enhancing effect of theophylline on E-rosette formation was not significant. The same was true for PHA stimulation; in 5 out of 7 cases the mitogen enhanced the stimulating effect of preincubation and had no significant effect on fresh lymphocytes from CLL patients. Preincubation significantly reduced the percentage of surface immunoglobulin positive B cells from CLL patients in all cases studied, and theophylline treatment had an additional effect on this phenomenon. No such effect of theophylline on fresh B cells from CLL patients could be observed. Preincubation had no significant effect on control lymphocytes. The effect of theophylline on control lymphocytes as compared to lymphocytes from CLL patients was completely different for T as well as for B lymphocytes. E-rosette formation from control lymphocytes (fresh and preincubated) was significantly inhibited in the presence of theophylline. No significantly enhanced responsiveness to PHA could be observed after treatment of fresh or preincubated lymphocytes with theophylline. Preincubation and theophylline treatment had no significant effect on the percentage of Ig positive B cells from control lymphocytes. The results suggest that under appropriate experimental conditions, preincubation and subsequent theophylline treatment can affect T as well as B cell markers on lymphocytes from CLL patients. These two manipulations seem to induce some degree of lymphocyte maturation in certain CLL cases, as indicated by increased E-rosette formation and PHA responsiveness for T cells and a decrease in surface Ig receptors for B cells.**

The therapeutic effect of theophylline, similar to other methylated xanthines, is associated with a number of cellular reactions which include translocation of intracellular calcium, blockade of receptors for adenosine and accumulation of cyclic AMP.[1]

It has been observed that theophylline can induce *in vitro* immunocompetence to noncompetent lymphocytes and can mimic the *in vitro* effect of thymic hormones. The biochemical mechanisms induced in activated cells by thymic hormones as well as by theophylline seem to be mediated mainly by cyclic AMP.[2] Theophylline *in vitro* was found to affect distinct T lymphocyte subpopulations according to their stage of differentiation and maturation and it was found to induce *in vitro* T cell maturation of human fetal liver, spleen and cord blood lymphocytes.[3-5]

In chronic lymphocytic leukemia (CLL) of the B cell type, many investigators[6-10] have reported structural and functional defects for T cells, in addition to B cell abnormalities. In this study, we tested the modulating effect of theophylline *in vitro* on lymphocytes from CLL patients. In the presence and in the absence of theophylline, the spontaneous binding capacity of T lymphocytes to sheep red blood cells and the phytohemagglutinin (PHA) response of lymphocytes were evaluated. For B cells fluorescent surface immunoglobulin staining was evaluated before and after *in vitro* treatment with theophylline.

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Theophylline was tested on fresh and on preincubated lymphocytes. According to various authors[11-14] preincubation has an enhancing effect on the immunological responsiveness of certain T lymphocytes. Preincubation, as well as subsequent *in vitro* treatment of lymphocytes with theophylline were found to improve significantly the E-rosette forming capacity and the PHA response of lymphocytes from CLL patients in 70% of the cases. In contrast to this, the percentage of cells from CLL patients stained for cytophilic immunoglobulin decreased markedly after preincubation. After theophylline treatment, some additional decrease could be observed in all five cases tested.

MATERIALS AND METHODS

Patients and control subjects

Sixteen patients (8 women and 8 men) and 11 healthy controls were studied in this work. The disease status according to Rai's clinical staging in CLL[15] was mild to moderate in 10 patients and more severe (stage II and III) in 6 patients. Ten patients were in peripheral remission (lymphocytes $<$ 25,000 cells/μl) and the lymphocyte count in the other 6 patients was from 27,000 to 92,000 cells/μl.

Lymphocyte preparation

Lymphocytes were separated from peripheral blood according to the method of Boyum.[16] Part of the fresh lymphocytes were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 at a concentration of 2×10^6 cells/ml, in culture medium RPMI 1640 supplemented with antibiotics and 10% pooled human serum.

B cell numbers

We assessed the number of B cells in fresh lymphocytes by determining the percentage of cells exhibiting surface immunoglobulin staining[17] with fluorescein conjugated polyvalent antihuman immunoglobulin (Meloy Lab., Springfield, IL, USA.)

E-rosettes

E-rosettes were tested according to Limatibul *et al*,[4] before and after *in vitro* treatment with theophylline (1-3 dimethylxanthine anhydrous crystals, Sigma, USA), at 2.5×10^{-3}M and 1.2×10^{-3}M. For preincubated lymphocytes lower concentrations of theophylline of 10^{-4}M and 0.25×10^{-4}M were used. Briefly, equal volumes of mononuclear cells (3×10^6/ml) and theophylline solutions were incubated for 2 hr at 37°C prior to addition of red blood cells. For fresh lymphocytes, E-rosettes were determined after 1 hr incubation at 4°C with sheep red blood cells (SRBC). For preincubated cells, they were determined twice : after 1 and after 24 hr incubation with SRBC. A count of 300 lymphocytes was made to determine the percentage of E-rosettes.

PHA stimulation

Theophylline was present in the culture medium for the entire period of cultivation, 5 days. This prolonged incubation time was observed by others and by us to be necessary for lymphocyte cultures from CLL patients to be stimulated by PHA.[18,19]

To avoid possible alterations of lymphocyte viability resulting from prolonged contact with the drug, lower concentrations of theophylline were used for PHA stimulated cultures of fresh or preincubated cells. Tritiated thymidine incorporation was measured and compared in PHA stimulated cultures with and without theophylline.

RESULTS

E-rosette forming T cells and surface immunoglobulin-positive B lymphocytes

The proportion of E-rosette forming cells in fresh lymphocytes from CLL patients was significantly reduced as compared to controls (18 ± 3.3 *vs.* 57 ± 1.7, Table 1). In contrast to control lymphocytes, overnight preincubation had a significant enhancing effect on E-rosette forming capacity of lymphocytes from 11 out of 14 CLL patients (Tables 1 and 2). The proportion of B lymphocytes in the CLL patients varied from 60% to 80% and in controls from 17% to 22% (Tables 1 and 2). Overnight incubation had a highly significant effect on surface immunoglobulin staining of B lymphocytes from all 5 CLL patients tested and the percentage of immunoglobulin positive cells decreased after incubation by 45 to 74% (Table 2). No such effect was observed after preincubation of lymphocytes from controls and the percentage of immunoglobulin positive cells was similar for fresh and preincubated lymphocytes (Table 2).

Effect of theophylline on E-rosette formation

The effect of theophylline on the E-rosette forming capacity of lymphocytes from CLL patients is summarized in Table 1. No significant enhancing effect of theophylline on E-rosette formation could be observed with fresh lymphocytes (Table 1). A significant enhancing effect of theophylline could be observed on E-rosette formation of preincubated lympho-

cytes in 11 out of 14 patients (Tables 1 and 2). The enhancing effect of theophylline was more evident at a concentration of 10^{-4}M and increased after prolonged incubation at 4°C (Table 1). Mean percentage ± SE of E-rosettes from fresh theophylline treated lymphocytes was 19% ± 5.7 *vs* 32.0 ± 5.2 for preincubated theophylline treated lymphocytes (Table 1).

Table 1 The *in vitro* effect of theophylline on the E-rosette forming capacity of fresh and preincubated lymphocytes from CLL patients as compared to controls

CLL lymphocytes					Control lymphocytes				
No. of cases	Theophylline concentration	B cells **(%) (mean ± SE)	E rosettes (%) (mean ± SE)		No. of cases	Theophylline concentration	B cells (%) (mean ± SE)	E rosettes (%) (mean ± SE)	
			1 hr	24 hr				1 hr	24 hr
			Fresh cells						
11	Untreated	68±2.4	18±3.3	–	10	Untreated	19±1.8	57±1.7	–
11	2.5×10^{-3}M		19±5.7 (p=NS)*	–	10	2.5×10^{-3}M		44±3.4 (p<0.05)	–
4	1.2×10^{-3}M		20±3.5 (p=NS)	–	4	1.2×10^{-3}M		45±4.5 (p=NS)	–
			Preincubated cells						
9	Untreated		25±3.1	28±4.1	7	Untreated		50±1.3	55±1.8
9	10^{-4}M		32±5.2 (p< 0.05)	37±5.1 (p< 0.01)	7	10^{-4}M		48±2.5 (p=NS)	46±2.0 (p< 0.05)
6	0.25×10^{-4}M		27±4.3*** (p = NS)	33±4.9*** (p = NS)	7	0.25×10^{-4}M		47±2.9 (p=NS)	47±1.8 (p< 0.01)

* Student's paired matched test between E rosette (%) from untreated as compared to theophylline treated cells.
** % of cells stained with fluorescent polyvalent antihuman immunoglobulin.
*** Compared to E rosette % of untreated lymphocytes from the same 6 patients, 26±3.3 and 28±4.5 after one hour and after 24 hours storage at 4°C, respectively.

Table 2 The *in vitro* effect of theophylline on fresh and preincubated B cells from CLL patients

CLL lymphocytes				Control lymphocytes		
No. of cases+	Theophylline concentration	E rosettes (%) (mean ± SE)	B cells (%)* (mean ± SE)	No. of cases	Theopylline concentration	B cells (%) (mean ± SE)
			Fresh cells			
5	Untreated	14±4.4**	58±4.9***	4	Untreated	19±2.2
5	2.5×10^{-3}M	–	54±3.8 (p=NS)	4	2.5×10^{-3}M	15±3.5 (p=NS)
5	1.2×10^{-3}M	–	57±4.9 (p=NS)	4	1.2×10^{-3}M	15±1.5 (p=NS)
			Preincubated cells			
5	Untreated	17±5.2**	23±3.7***	4	Untreated	19±3.1
5	10^{-4}M	–	19±3.5 (p< 0.01)	4	10^{-4}M	18±3.5 (p=NS)
5	0.25×10^{-4}M	–	18±1.9 (p = NS)	4	0.25×10^{-4}M	17±1.2 (p = NS)

+Additional cases not included in Table 1
*See footnotes to Table 1
For CLL ***E rosettes fresh *vs* preincubated P < 0.05 (24 hr incubation at 4°C)
***B fresh untreated *vs* B preincubated untreated p < 0.01

Preincubation as well as addition of theophylline were more effective in the enhancement of E-rosette formation in CLL patients in stage 0 and I, as compared to patients in stage II and III. The effect of theophylline on E-rosette formation of control lymphocytes (fresh or preincubated) was completely different from that for lymphocytes from CLL patients (Table 1). Similar to other investigators, we found that theophylline had an inhibitory effect on E-rosettes from control lymphocytes, on " theophylline sensitive " T cells.[4,5] This inhibitory effect was observed on fresh as well as on preincubated control lymphocytes and was more evident after prolonged incubation of the lymphocytes with SRBC (Table 1).

Effect of theophylline on PHA stimulation

The effect of theophylline on the PHA response of lymphocytes from CLL patients is summarized in Table 3. The stimulation rate of lymphocytes from CLL patients was significantly lower than for controls (Table 3). Similar to E-rosette formation, theophylline enhanced the PHA response of preincubated lymphocytes from CLL patients. This effect was observed in 5 out of 7 cases in a range from 15% to 50%. Theophylline had no significant effect on PHA stimulation of fresh or preincubated control lymphocytes (Table 3).

Effect of theophylline on surface immunoglobulin positive B lymphocytes

The effect of theophylline on the percentage of immunoglobulin positive staining B lymphocytes from CLL patients is summarized in Table 2. In all 5 patients tested after theophylline treatment of preincubated cells, an additional decrease of immunoglobulin positive stained cells (ranging from 12% to 43%) could be observed. No similar consistent effect of theophylline could be observed on fresh lymphocytes from CLL patients or on control lymphocytes (fresh and preincubated).

DISCUSSION

The results of this study suggest that, in certain cases of B cell CLL, *in vitro* preincubation and subsequent addition of theophylline may induce abnormal lymphocytes to acquire a certain degree of maturation as expressed by T and B cell markers, by enhanced E-rosette forming capacity, by increased responsiveness to PHA and by a decreased percentage of surface immunoglobulin staining B cells. The effects of these two manipulations, incubation and theophylline treatment, were additive for T as well as for B cells.

The mechanism of activation of preincubated as compared to fresh lymphocytes from CLL

Table 3 The *in vitro* effect of theophylline on the proliferative response to PHA of fresh and preincubated lymphocytes from CLL patients

CLL lymphocyte cultures (5 days)*			Control lymphocyte cultures (5 days)*		
No. of** cases	Theophylline concentration	Mean ± SE (cpm×10^3)	No. of cases	Theopylline concentration	Mean ± SE (cpm × 10^3)
		Fresh cells			
6	Untreated	14.7±5	6	Untreated	42±4.8
6	10^{-4}M	12.0±3.5 (p=NS)	6	10^{-4}M	47±3.8 (p=NS)
6	0.25 × 10^{-4}M	14.5±4.5 (p=NS)	6	0.25 × 10^{-4}M	44°7.4 (p=NS)
		Preincubated cells			
7	Untreated	22.9±8.0	6	Untreated	50±12.7
7	10^{-4}M	25.2±7.9 (p< 0.05)	6	10^{-4}M	54±17.8 (p=NS)
7	0.25 × 10^{-4}M	26.5±9.5 (p < 0.05)	6	0.25 × 10^{-4}M	55±17.2 (p = NS)

**The difference between parallel samples (triplicates) was within the range of 5%

**PHA 10 μg/ml culture.

**Mean values for unstimulated cultures (with and without theophylline) were between 0.5 and 0.7 × 10^3 cpm.

**Cases included in Table 1.

patients is not clear. It is suggested that previous depletion of some short lived suppressor cell,[11] or direct regulation of the responder cells themselves by preincubation[14] may be necessary for lymphocytes from CLL patients to improve their capacity to form E-rosettes and to respond to PHA.

In this connection, it may be mentioned that significant changes in Fc IgG and IgM membrane receptor positive T cells were observed after 24 hours of incubation of lymphocytes from CLL patients as well as of controls.[20] However, the effect of lymphocyte preincubation for these two groups was different. A less dramatic increase in Fc IgM positive cells and a somewhat increased disappearance of Fc IgG (T_γ) positive cells could be observed after incubation of lymphocytes from CLL patients as compared to controls.

The relationship between T cells and malignant B cells in CLL patients has been studied by various authors.[21-23] It has been found that the cooperative potential of Fc IgG cells (which include suppressor cells) and Fc IgM T lymphocytes (which include T helper cells) is defective for *in vitro* immunoglobulin production in CLL patients as compared to controls.[21,22] It may be suggested that the marked decrease in immunoglobulin positive stained B cells from CLL patients observed by us may be correlated to specific changes in T cell subpopulations after preincubation. On the other hand, it is also possible that incubation itself, as with T cells, may specifically affect Ig surface receptors on B cells from CLL patients.

It is possible that the differences in cell surface markers between fresh and preincubated T and B lymphocytes from CLL patients observed in this study are correlated to cell cycle changes in CLL lymphocytes which can affect surface molecule fluctuations.[24] It is also suggested that only the effect of theophylline on preincubated cells may be correlated to such cell cycle changes. However, the mechanism of the immunomodulating effect of theophylline on lymphocyte markers of T and B cells from CLL patients is not clear. Our observed case to case variability of immunomodulation by theophylline, especially for E-rosette and PHA stimulation, may suggest a possible correlation with different maturation levels of T cell subsets. Subpopulations of human T lymphocytes differing in their stages of maturation have been found to differ in their cAMP contents; less mature T lymphocytes have lower cAMP contents and reduced responsiveness to PHA as compared to more mature T lymphocytes.[25] By inducing increased cAMP levels, theophylline was found to inhibit the release of calcium from intracellular store sites and to affect in this way the migrating capacity of peritoneal macrophages from guinea pigs.[26]

On human melanoma cells theophylline was found to induce modulation of susceptibility to natural killer cell mediated cytolysis with clonal selection for a specific cell type.[24]

In conclusion, this study provides evidence that preincubation as well as subsequent theophylline treatment of lymphocytes from CLL patients may in certain cases significantly reduce the percentage of Ig positive B cells, increase the E-rosette forming capacity of T lymphocytes and improve to some degree the responsiveness to PHA of cultured cells.

Although, at this stage, experiments have not explored other lymphocyte functions, the system does offer a model for further *in vitro* investigations of the immunomodulating effect of theophylline on lymphocytes from CLL patients.

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